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Award Number DAMD17-98-1-8023

TITLE: Alternative DNA Damage Checkpoint Pathways in Eukaryotes

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REPORT DATE: April 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

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# REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank	April 1999	Annual Summary (23 Mar 9				
4. TITLE AND SUBTITLE Alternative DNA Damage Check		5. FUND	ING NUMBERS 17-98-1-8023			
6. AUTHOR(S) Yi-Chen J. Li, Ph.D.						
7. PERFORMING ORGANIZATION NA Baylor College of Medicine Houston, Texas 77030-3498		DRMING ORGANIZATION RT NUMBER				
9. SPONSORING / MONITORING AG U.S. Army Medical Research an Fort Detrick, Maryland 21702-56	d Materiel Command		NSORING / MONITORING NCY REPORT NUMBER			
11. SUPPLEMENTARY NOTES						
12a. DISTRIBUTION / AVAILABILITY Approved for Public Release; Di	STATEMENT stribution Unlimited	12b. DIS	STRIBUTION CODE			
The goals of this grant are to perform a mutagenesis screen to identify the genes in the alternative DNA damage checkpoint pathway in yeast, to characterize and clone these genes, and to isolate and analyze the human counterparts of these genes. During the first year of this grant, we have constructed a $cdc9-8$ , $rad9_{\Delta}$ double mutant strain SCP2, which is both temperature-sensitive at 30°C and UV-sensitive, for the mutagenesis screen. Approximately 220,000 colonies of SCP2 have been mutagenized by EMS and screened for temperature-sensitivity at 30°C. This primary screen yields three temperature-sensitive mutants, $chb13$ , $chb16$ , and $chb57$ . A secondary UV sensitivity screen was also performed on these three $chb$ mutants. In summary, $chb13$ is a strong mutant that can no longer be rescued by $CHES1$ in both temperature- and UV-sensitivity. The other two mutants have only one strong phenotype. Specifically, $chb16$ is highly temperature-sensitive but only moderately UV-sensitive, whereas $chb57$ is very sensitive to UV but not as sensitive to high temperature. Our next goal is cloning the genes mutated in these three strains.						
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 7			
			16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited			

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## Progress Report (March 1998 to March 1999)

### **Technical Objective 1-1**

Perform a comprehensive mutagenesis screen in S. cerevisiae to isolate yeast mutant strains which have defective checkpoint bypass genes

Our working hypothesis is that CHESI suppresses the radiation sensitivity of mecI-1 and  $rad9\Delta$  by activation of an alternative DNA damage-induced G2 checkpoint pathway in S. cerevisiae. The genes in the pathway can be identified genetically. The ultimate objectives of this grant proposal are to identify, characterize, and clone the genes in this alternative pathway, and to isolate the human homologs of these genes and analyze their structure and expression in human breast cancer derived cell lines and human breast tumor samples. The goals of the first year of this grant are to develop a temperature sensitive strain, to optimize mutagenesis conditions, and to accomplish a mutagenesis screen to identify mutant strains that can no longer be rescued by CHESI in the absence of wild type RAD9.

The method proposed in this project utilizes a temperature sensitive strain that does not grow at 30°C and is sensitive to UV irradiation. This requires the combination of cdc9-8 and  $rad9\Delta$  mutations in the same genome. The  $rad9\Delta$  not only lowers the permissive temperature of cdc9-8 from 30°C to 23°C, but also provides the UV sensitive phenotype. The cdc9-8 gene was gap repaired out from the 9085-1-10-4 strain and put onto an integration vector with URA3 marker. The plasmid was then introduced into a  $rad9\Delta$  strain (Y438 from the Elledge's laboratory) and plated onto URA deficient- and 5-FOA media sequentially. This allowed isolation of a strain where the cdc9-8 allele replaces the wild type CDC9 to generate the double mutant strain SCP2.

Different amount of EMS, ranging from 0 to 50 µl, was tested to assay for optimal mutagenesis. We chose 5 µl for it gave approximately 50% killing in SCP2. The mutagenesis screen was carried out by a slightly modified method to the one originally described in the application. Specifically, SCP2 was transformed with two plasmids, each carrying CHES1 or RAD9 genes, before EMS mutagenesis. After exposure to EMS, the cells were washed and plated onto selective medium at 30°C. Since the cells still contain wild type RAD9 at this point, they are able to grow at 30°C. The mutants were replica-plated to 5-FOA to select against the RAD9 plasmid and screened for growth at 23°C but not at 30°C. The tentative name assigned to the qualified mutants is chb for checkpoint bypass. The colonies identified on this first pass were then patched and replica-plated to test for temperature sensitivity more carefully at 37°C, 32°C, 30°C, and room temperature. The clones, which no longer grew at 30°C from the second temperature sensitivity test, were subject to a secondary screen to determine if they have also lost the response of CHES1 after UV irradiation. Our model predicts that checkpoint bypass gene mutants should be UV sensitive even when CHES1 is present in this rad9\Delta strain. The UV sensitivity screen was performed by quantifying the survival rate after exposure to 10 J/m<sup>2</sup> of UV radiation, in comparison to the survival rate of the parental strain transformed with either empty vector or CHES1-containing plasmid.

We have mutagenized and screened approximately 220,000 clones and 310 putative temperature sensitive clones were picked originally. The majority of clones

failed on the second temperature sensitivity test, leaving three strong candidates that are truly temperature sensitive in the presence of CHES1 when the RAD9 plasmid was lost. Among those, chb13 and chb16 are highly temperature sensitive whereas chb57 is a weaker allele. With regard to UV sensitivity, chb13 and chb57 are also sensitive to UV radiation but the effect of chb16 is intermediate when compared to the controls (figure 1). The growth of chb16 is slower than the other two chb strains, therefore, chb16 is a relatively unhealthy mutant in general. Overall, chb13 appears to be a very strong mutant that has lost all response to CHES1 by our assays. Both chb16 and chb57 are moderate mutants that have one strong phenotype. The results of this mutagenesis screen are summarized in table 1. Construction of a cdc9-8 and  $rad9\Delta$  double mutant strain of the opposite mating type is underway in order to perform complementation analysis to determine whether the mutants are dominant or recessive. Once defined, we will clone the genes that were recessively mutated in the chb strains by introducing a S. cerevisiae genomic library into the mutant strain and selecting for growth at  $30^{\circ}C$  in the presence of CHES1.

Our previous results showed that a *chk1* mutant is at least partially defective in the response to *CHES1* (D. Pati, unpublished data). This suggests that *CHK1* maybe a candidate gene in the proposed alternative pathway. We will test all three *chb* mutants for the complementation by *CHK1*. Disruption of *CHK1* in the parental strain SCP2 is also underway for the comparison of the phenotypes with these *chb* mutants.

The analysis of these mutants has also allowed us to confirm that the CHES1-dependent pathway is partially parallel to the RAD9-dependent pathway. Our previous data showed that CHES1 could either act on a gene product downstream of DUN1 in the primary MEC1-dependent pathway or, more likely, activate an alternative pathway (Pati et al., 1997). Consistent with the parallel pathway model, introduction of a wild type RAD9 gene restores UV resistance to the SCP2 strain and all three mutants. Since the chb mutants are UV resistant when RAD9 is present on the episome, they are apparently not simply downstream of RAD9. This favors the alternative parallel pathway model. Our current model for the CHES1 activated checkpoint is demonstrated in figure 2.

In the "Statement of Work" attached to the original proposal, the task for the first year was to finish both the primary temperature sensitivity screen and the secondary UV sensitivity screen. These have been completed.

### Reference

D. Pati, C. Keller, M. Groudine, and S. E. Plon. 1997. Reconstitution of a *MEC1*-independent Checkpoint in Yeast by Expression of a Novel Human *fork head* cDNA. Mol. Cell. Biol. 17: 3037-3046.

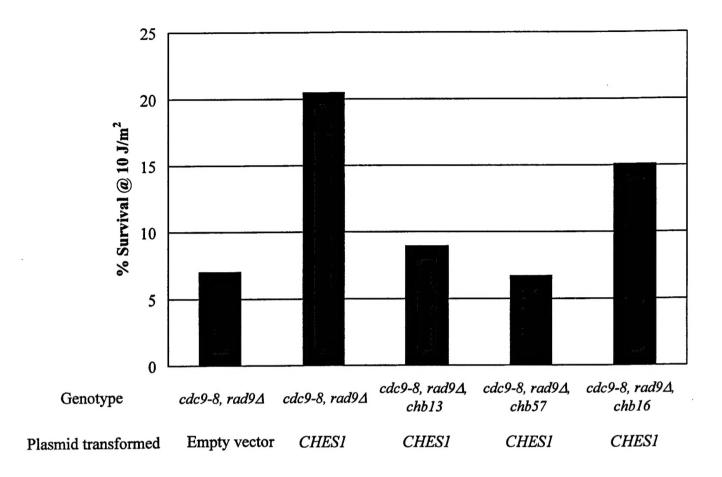


Figure 1 – Results of secondary assay of three *chb* mutants identified in the large-scale mutagenesis screen. The percent survival after exposure to  $10 \text{ J/m}^2$  is shown for the parental  $rad9\Delta$  strain with a control vector or *CHES1* and the three *chb* mutant strains with *CHES1*.

Strain	Growth at 30°C	Survival after 10 J/m <sup>2</sup> UV irradiation
rad9∆, cdc9-8 + Vector	•	7.0%
rad9∆, cdc9-8 + CHES1	+++	20.5%
rad9∆, cdc9-8, chb13 + CHES1	-	8.9%
rad9∆, cdc9-8, chb57 + CHES1	+	6.7%
rad9∆, cdc9-8, chb16 + CHES1	•	15.1%

Table 1 – Summary of both primary and secondary screens for mutants obtained in the mutagenesis screen.

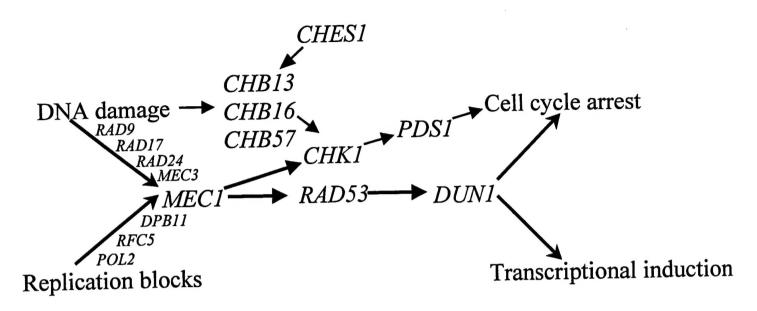


Figure 2 – Current Model of CHES1 Dependent Checkpoint Pathway.